J Antimicrob Chemother 2016; **71**: 2386–2396
doi:10.1093/jac/dkw156 Advance Access publication 12 May 2016

Journal of Antimicrobial Chemotherapy

ECIL guidelines for the diagnosis of *Pneumocystis jirovecii* pneumonia in patients with haematological malignancies and stem cell transplant recipients

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The Fifth European Conference on Infections in Leukaemia (ECIL-5) convened a meeting to establish evidence-based recommendations for using tests to diagnose *Pneumocystis jirovecii* pneumonia (PCP) in adult patients with haematological malignancies. Immunofluorescence assays are recommended as the most sensitive microscopic method (recommendation **A-II**). Real-time PCR is recommended for the routine diagnosis of PCP (**A-II**). Bronchoalveolar lavage (BAL) fluid is recommended as the best specimen as it yields good negative predictive value (**A-II**). Non-invasive specimens can be suitable alternatives (**B-II**), acknowledging that PCP cannot be ruled out in case of a negative PCR result (**A-II**). Detecting β-D-glucan in serum can contribute to the diagnosis but not the follow-up of PCP (**A-II**). A negative serum β-D-glucan result can exclude PCP in a patient at risk (**A-II**), whereas a positive test result may indicate other fungal infections. Genotyping using multilocus sequence markers can be used to investigate suspected outbreaks (**A-II**). The routine detection of dihydropteroate synthase mutations in cases of treatment failure is not recommended (**B-II**) since these mutations do not affect response to high-dose co-trimoxazole. The clinical utility of these diagnostic tests for the early management of PCP should be further assessed in prospective, randomized interventional studies.

Introduction

Pneumocystis jirovecii is a ubiquitous fungus that is specific for humans and responsible for *P. jirovecii* pneumonia (PCP) of patients with T cell deficiencies, including those with HIV infection, solid organ transplant recipients and patients with cancer, as well as adults and children with other underlying immunological diseases.[1,2] The laboratory diagnosis of PCP has evolved rapidly in the last few years. Historically, diagnosis was based on tinctorial or immunofluorescent staining of bronchoalveolar lavage (BAL) fluid samples. New diagnostic approaches include the use of alternative clinical specimens, such as induced sputum, and the detection of new biological markers, namely fungal DNA and antigens. Primarily developed for patients with HIV infection, the interpretation of these new diagnostic tests did not appear straightforward for diagnosing PCP in other patients. Indeed, the

clinical presentation of patients with HIV and other immunocompromised patients is clearly different.[1,2] Similarly, the fungal load responsible for the development of PCP may also differ in these populations.[3–5] There is also no current guideline for interpreting the results of these diagnostic tests in haematology.

The goal of the European Conference on Infections in Leukaemia (ECIL) is to develop recommendations for managing infectious complications associated with the treatment of haematological malignancies. Furthermore, there are few consensus guidelines for using diagnostic tests or procedures to diagnose fungal diseases. The third ECIL (ECIL-3) was a first attempt to obtain a consensus on the diagnosis of aspergillosis, candidiasis and cryptococcosis.[6–10] The objective of ECIL-5 was to assist teams involved in managing patients with haematological malignancies to diagnose PCP. Two groups of clinicians and microbiologists undertook a systematic literature review based on a search strategy up to 31 August 2013. Recommendations were graded on the basis of the quality of evidence, and are summarized in Table 1.[1] This grading system was designed to make therapeutic recommendations and may not be appropriate for biological tests. For instance, an A-I level of recommendation is not possible since level I requires the results of randomized controlled studies. However, a grading system specifically designed for diagnostic tests is not currently available. Moreover, the grading system is familiar to clinicians and laboratory personnel alike because ECIL has used it since 2009.

The slide sets of ECIL-5 have been available since April 2014 (http://www.kobe.fr/ecil/telechargements2013/ECIL5%20-%20PjP%20guidelines-%20Biology.pdf).

Search strategy and selection of publications

A systematic literature review was based on a search strategy in the PubMed and Scopus databases for English language literature published up to 31 August 2013 based on the following MeSH terms: ‘polymerase chain reaction NOT real-time polymerase chain reaction AND pneumonia, *Pneumocystis* AND humans NOT genotype NOT drug resistance, microbial’; ‘real-time polymerase chain reaction AND pneumonia, *Pneumocystis* AND humans’; ‘real-time polymerase chain reaction AND pneumonia, *Pneumocystis* AND humans’; ‘immunofluorescence AND pneumonia, *Pneumocystis* AND humans’.

Our electronic search on diagnosis of PCP retrieved 153 publications on immunofluorescence (IF) staining, 280 on conventional PCR assays and 39 on quantitative real-time PCR (qPCR) assays. A total of 31 publications dealing with more than 5875 BALs, of which 2402 (41%) were from patients without HIV infection, were selected as providing 35 comparisons between microscopy and PCR assays and descriptions of the patients tested (Table 2).[3,4,11–39]

Biological diagnosis

Owing to the non-specificity of imaging and clinical signs and the frequent co-infections and non-infectious causes of lung infiltrates, together with the possible toxicity and the long duration of PCP therapy,[1,2,40] making a PCP diagnosis on clinical criteria or imaging alone is not recommended.[41] In general and in haematology in particular, it is of the utmost importance to detect and identify the pathogen involved in the infectious process.

Biological diagnosis using microscopy

P. jirovecii replicates asexually by binary fission of trophic forms (formerly trophozoites) and sexually, resulting in the formation of an ascus (formerly cyst) containing eight ascospores.[42] Since *P. jirovecii* is not cultivable *in vitro*, the diagnosis of PCP has long relied on microscopic detection of trophic forms and cysts using different stains [Giemsa (GS), toluidine blue O (TBO), calcofluor white (CW) and Gomori methenamine silver (GMS)]. Overall, the diagnostic performance of conventional stains is comparable. These stains were supplanted in the mid-1990s by direct or indirect IF using anti-*P. jirovecii* monoclonal antibodies. Immunofluorescence assays use mainly anti-cyst antibodies and, more rarely, a mixture of

Table 1. Recommended diagnosis of PCP in adult patients with haematological malignancies and stem cell transplant recipients (it is not recommended that PCP diagnosis should rely only on clinical criteria or imaging)

Specimen/technique	Recommended usage	Strength of recommendation	Quality of evidence
Diagnostic specimen			
BAL fluid	allows detection of multiple aetiologies	A	II
other (non-invasive specimens[a])	alternative specimen to BAL	B	II
Diagnostic technique			
Respiratory samples			
immunofluorescence assays	most sensitive microscopic diagnostic method	A	II
real-time quantitative PCR	routine diagnosis allowing quantification	A	III
	exclusion of PCP by negative result in BAL only	A	II
Serum			
β-D-glucan	detection in serum as a contributive diagnostic tool	A	II
	exclusion of PCP by negative result	A	II
genotyping using multilocus sequence markers	investigation of suspected outbreaks	A	II
detection of dihydropteroate synthase mutations	not recommended in case of treatment failure	B	II

[a]Includes induced sputa, sputa and upper respiratory samples (nasopharyngeal aspirates, nasal or oral washes).

Table 2. Characteristics of the studies selected for analysis of PCR performance compared with microscopy (MS) in BAL fluid specimens

Reference	Publication year	BAL fluid (*n*)	HIV− samples (%)	MS type	PCR format	PCR target gene	MS+ (%)	PCR+ (%)	PCR+ MS+ (%)	PCR− MS+ (%)	PCR+ MS− (%)	PCR− MS− (%)
4	2012	353	87	IIFA	qPCR	mtLSU	17 (4.8)	80 (22.7)	17 (4.8)	0 (0)	63 (17.8)	273 (77.3)
34	2012	105	NA	DIFA	qPCR	mtLSU	9 (8.6)	27 (25.7)	9 (8.6)	0 (0)	18 (17.1)	78 (74.3)
3	2011	163	71	IIFA	qPCR	mtLSU	12 (7.4)	42 (25.8)	12 (7.4)	0 (0)	30 (18.4)	121 (74.2)
35	2011	83	91.8	DIFA	qPCR	mtLSU	13 (15.7)	20 (24.1)	12 (14.5)	1 (1.2)	8 (9.6)	62 (74.7)
32	2009	186	86.2	CW, TBO	qPCR	kex	21 (11.3)	33 (17.7)	21 (11.3)	0 (0)	12 (6.5)	153 (82.3)
37	2008	400	78.2	DIFA	qPCR	MSG	31 (7.8)	66 (16.5)	31 (7.8)	0 (0)	35 (8.8)	334 (83.5)
14	2008	133	0	GMS	qPCR	HSP70	62 (46.6)	67 (50.4)	61 (45.9)	1 (0.8)	6 (4.5)	65 (48.9)
31	2008	118	0	IIFA	qPCR	MtLSU	25 (21.2)	41 (34.7)	25 (21.2)	0 (0)	16 (13.6)	77 (65.3)
39	2006	214	NA	CW	qPCR	Cdc2	22 (10.3)	28 (13.1)	22 (10.3)	0 (0)	6 (2.8)	186 (86.9)
38	2005	53	NA	DIFA	qPCR	Tubulin	8 (15.1)	24 (45.3)	8 (15.1)	0 (0)	16 (30.2)	29 (54.7)
36	2004	150	87.3	GMS	qPCR	MSG	11 (7.3)	32 (21.3)	11 (7.3)	0 (0)	21 (14)	118 (78.7)
33	2003	529	18.9	IIFA	qPCR	MtLSU	35 (6.6)	81 (15.3)	35 (6.6)	0 (0)	46 (8.7)	448 (84.7)
range qPCR							4.8–46.6	13.1–50.4	4.8–45.9	0–1.2	2.8–30.2	48.9–86.9
22	2012	102	35.3	DIFA	nPCR	MtLSU	53 (52)	91 (89.2)	53 (52)	0 (0)	38 (37.3)	11 (10.8)
25	2000	146	0	NA	nPCR	ITS	71 (48.6)	89 (61)	71 (48.6)	0 (0)	18 (12.3)	57 (39)
	2000	257	67	GMS, GS	nPCR	MtLSU	29 (11.3)	56 (21.8)	29 (11.3)	0 (0)	27 (10.5)	201 (78.2)
30	1997	312	41.8	IIFA	nPCR	18S rRNA	40 (12.8)	45 (14.4)	39 (12.5)	1 (0.3)	6 (1.9)	266 (85.3)
29	1997	210	83.3	IIFA	nPCR	MtLSU	13 (6.1)	49 (23)	13 (6.2)	0 (0)	36 (17.1)	161 (77)
18	1997	127	17.3	GMS, GS	nPCR	MtLSU	25 (19.7)	50 (39.4)	25 (19.7)	0 (0)	25 (19.7)	77 (60.6)
28	1997	128	0	TBO	nPCR	MtLSU	34 (26.6)	38 (29.7)	31 (24.2)	3 (2.3)	7 (5.5)	87 (68)
26	1995	117	0	DIFA, GS	nPCR	18S rRNA	40 (34.2)	34 (29.1)	34 (29.1)	6 (5.1)	0 (0)	77 (65.8)
range nPCR							6.1–52	14.4–89.2	6.1–52	0–5.1	0–37.3	10.8–85.3
22	2012	102	35.3	DIFA	sPCR	MtLSU	53 (52)	69 (67.6)	53 (52)	0 (0)	16 (15.7)	33 (32.4)
15	2009	235	100	GS	sPCR	MtLSU	60 (25.5)	75 (31.9)	60 (25.5)	0 (0)	15 (6.4)	160 (68.1)
14	2008	136	0	GMS	sPCR	MtLSU	62 (45.6)	85 (62.5)	61 (44.9)	1 (0.7)	24 (17.6)	50 (36.8)
23	2003	192	0	GMS, GS	sPCR	MtLSU	51 (26.6)	70 (36.5)	51 (26.6)	0 (0)	19 (9.9)	122 (63.5)
23	2003	192	0	GMS, GS	sPCR	DHPS	51 (26.6)	70 (36.5)	51 (26.6)	0 (0)	19 (9.9)	122 (63.5)
21	2002	81	67.9	TBO, GS	sPCR	5S rRNA	24 (29.6)	35 (43.2)	24 (29.6)	0 (0)	11 (13.6)	46 (56.8)
20	2000	259	67	GMS, GS	sPCR	MtLSU	26 (10)	34 (13.1)	26 (10)	0 (0)	8 (3.1)	225 (86.9)
11	1998	112	NA	DIFA	sPCR	18S rRNA	17 (15.2)	19 (17)	17 (15.2)	0 (0)	2 (1.8)	93 (83)
18	1997	127	17.3	GMS, GS	sPCR	MtLSU	25 (19.7)	42 (33.1)	24 (18.9)	1 (0.8)	18 (14.2)	84 (66.1)
16	1995	137	0	NA	sPCR	MtLSU	54 (39.4)	53 (38.7)	45 (32.8)	9 (6.6)	8 (5.8)	75 (54.7)
17	1995	74	67.4	DIFA	sPCR	MtLSU	11 (14.9)	12 (16.2)	11 (14.9)	0 (0)	1 (1.4)	62 (83.8)
12	1994	154	45.4	DIFA	sPCR	MtLSU	21 (13.6)	22 (14.3)	21 (13.6)	0 (0)	1 (0.6)	132 (85.7)
19	1994	65	27.3	IIFA, GS	sPCR	MtLSU	27 (41.5)	27 (41.5)	27 (41.5)	0 (0)	0 (0)	38 (58.5)
24	1993	52	7.7	IIFA	sPCR	MtLSU	26 (50)	35 (67.3)	26 (50)	0 (0)	9 (17.3)	17 (32.7)
13	1991	71	0	IIFA	sPCR	MtLSU	37 (52.1)	32 (45.1)	31 (43.7)	6 (8.5)	1 (1.4)	33 (46.5)
range sPCR							10–52.1	13.1–67.6	10–52	0–8.5	0–17.6	32.4–86.9
range all PCR							4.8–52.1	13.1–89.2	4.8–52	0–8.5	0–37.3	10.8–86.9

CW, calcofluor white staining; DIFA, direct immunofluorescence assays; GMS, Gomori methenamine silver; GS, Giemsa staining; IIFA, indirect immunofluorescence assays; nPCR, nested endpoint PCR; sPCR, single endpoint PCR; TBO, toluidine blue O; NA, not available.

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anti-cyst and anti-trophic form antibodies. Immunofluorescence techniques were tested on BAL samples in 18/29 (62.1%) publications.[3,4,11–13,17,19,22,24,26,29–31,33–35,37,38]

The sensitivity of IF, irrespective of the technique used (direct or indirect), was better than conventional staining (recommendation **A-II**). However, GS and direct IF kits that allow trophic form detection were only used in 6/29 (20.7%)[18–21,23,26] and 9/29 (31%) publications,[11,12,17,22,26,34,35,37,38] respectively. Since a 1:10 ratio exists between asci and trophic forms,[43] we recommend the use of a combination of two stains, one to detect 'cysts' (anti-cyst IF assay is the most sensitive, compared with conventional staining using TBO, CW or GMS) and another to detect 'trophozoites' (GS) (**A-III**). The combination of techniques allows detection of both forms and evaluation of the ascus/trophic form ratio. The clinical relevance of this ratio is unknown. However, the normal ascus/trophic form ratio of 1:10 decreases after anti-PCP prophylaxis and treatment with tetracycline or macrolide antibiotics.[43,44]

Biological diagnosis using PCR

Diagnostic PCR format

It was immediately obvious after the first publication that PCR for PCP[45] had the potential to be more sensitive than IF in detecting *P. jirovecii*. However, this raised the issue of interpretation of discordances between clinical evaluation and PCR results, in particular for a PCR-positive but IF-negative sample. In a recent review,[46] reported specificities for PCP diagnosis using qPCR assays ranged between 83% and 100%, mainly because some *P. jirovecii* DNA-positive patients with negative microscopy were not considered as having PCP. On the other hand, sensitivities ranged from 82% to 100%, suggesting that some clinicians consider some pneumonia as PCP even in the absence of any detected *P. jirovecii* DNA. Therefore, false-positive and false-negative PCR results can result from insufficient technical performance, discussed below, but also from the clinical definition of the diagnostic.

In the two recent meta-analyses, qPCR assays gave better results than non-qPCR assays in terms of sensitivity and specificity.[47,48] This can be explained by technical issues such as better prevention of false-positive results due to the closed format of the qPCR assays, which prevents contamination with amplicons, the main source of false positives. Moreover, there are now clear guidelines for the validation of the qPCR assays (minimum information for publication of quantitative real-time PCR experiments, MIQE), which results in more homogeneous and comparable assays.[49] These MIQE guidelines insist on the analytical validation of the test, with a clear delineation of the limit of detection and the amplification yield. An internal control (IC) of the amplification is also mandatory. This IC is intended for monitoring of the PCR yield, and then the presence of PCR inhibitors, in each clinical sample and to check that the yield obtained during validation of the PCR is the same as the one in the clinical sample. This last point is crucial to avoid false-negative results. Since human DNA is present in large but variable quantities, the IC cannot consist of a human gene. Any failure in correct amplification of the IC should be interpreted as an inconclusive result and testing should be repeated.

The quantitative expression of the results is of utmost importance for interpreting the qPCR results since, for a given fungal load, a patient may be asymptomatic or, conversely, seriously ill.[3,35] Moreover, there is a density gradient of the fungus from the alveoli to the oral cavity, with the highest concentration being found in the alveoli. Since different quantities are expected, the different clinical specimens cannot be judged identical. Therefore, if the main goal is to reliably quantify the fungal load and avoid false-positive results, qPCR with the integration of adequate controls is the recommended PCR format (**A-III**). Moreover, this is the only format that can really meet the requirements for a routine diagnostic test, namely reliability, reproducibility, ease of application, cost-effectiveness and quick turnaround time. A recent comparison of three centres demonstrated the transferability of the quantitative results with correlation coefficients >0.84.[50] Comparisons of homemade and commercial qPCR assays were performed, showing that commercial assays were not superior.[51–54]

Respiratory specimens

BAL fluid has been the most studied specimen for investigation of pneumonia in immunocompromised patients,[55] so it is the preferred specimen (**A-II**). However, since BAL requires dedicated personnel and is semi-invasive, it is not always possible to obtain the specimen. Consequently, upper respiratory specimens (URSs) have been evaluated, including induced sputum (IS), oral washings (OW), nasopharyngeal aspirate (NPA) and nasal swabs (NS). Since *P. jirovecii* lives and thrives at the surface of the alveolar cells, mainly on type I pneumocytes, a gradient is expected with the highest fungal load in BAL fluids and the lowest loads in OW or NPA. This obviously impacts on the sensitivity of the diagnostic procedure since the chances of detection are reduced. This will inevitably affect the clinical value of any test done with the specimen.

Although strict comparisons are scarce,[56–59] agreement on the yield of the different specimens (BAL > IS and IS > URS) is possible. For PCR in IS, a higher sensitivity was reported in patients with HIV infection, which is consistent with the higher fungal load.[3] For other immunocompromised patients, there is a need to deduce the alveolar load according to the IS or lower respiratory specimens' quantitative results. A positive result in IS or lower respiratory specimens probably indicates a much higher load in the alveoli. A negative result in IS or lower respiratory specimens incurs the risk of missing the diagnosis, more particularly in patients that are HIV negative, who tend to have PCP associated with low fungal load.[3,60,61]

Interpretation of qualitative and quantitative results (Figure 1)

The first challenge is how to express quantitative results. Authors who use a plasmid for validation and calibration presented the results as copy/volume. However, some authors use the crude qPCR result [quantification cycle (Cq), cut-off threshold (Ct) or crossing point (Cp)], whereas others translate it into the number of microorganisms. The availability of an international standard could solve this issue but one is currently not available. For IF-positive samples, qPCR gave results similar to those given by optical evaluation of the fungal load (number of cysts, often semi-quantitatively expressed as +, ++ or +++).[32,60]

The situation where IF is positive corresponds to PCP (**A-II**) regardless of the type of specimen. There is an additional value of qPCR in this setting. qPCR allows confirmation of the microscopic examination, since false-positive microscopy results have

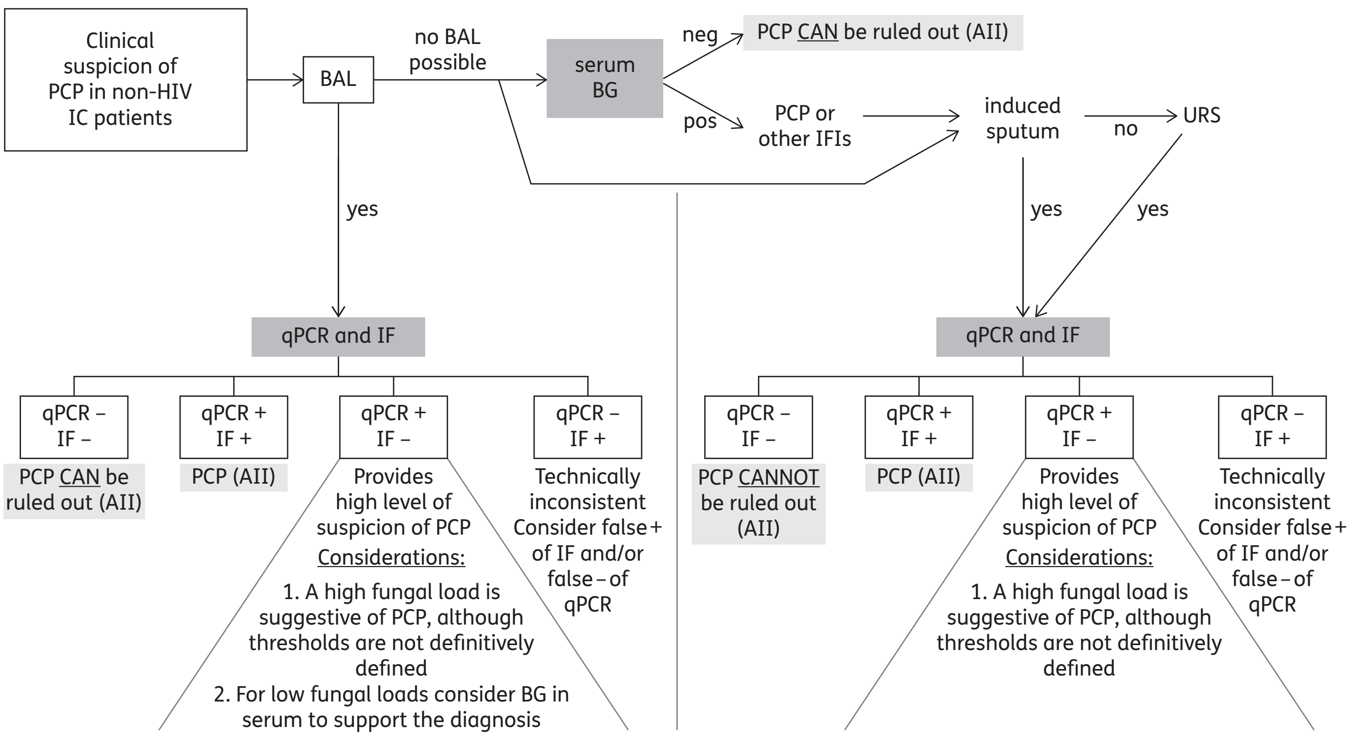

Figure 1. Flow chart for the diagnosis of *Pneumocystis* pneumonia in non-HIV immunocompromised (IC) patients. Biological tests are highlighted in dark grey and recommendations in light grey. BG, β-D-glucan; A-II, level of recommendation; IFI, invasive fungal infection.

been reported.[62–64] Moreover, there is an overlap in the sensitivity limit of IF, with some IF-negative and PCR-positive samples containing a higher *P. jirovecii* DNA content than some IF-positive samples. For instance, with IF as the standard, the qPCR assay sensitivity was 100% for $>2.6\ \log_{10}$ copies/μL and the specificity was 100% for $>4\ \log_{10}$ copies/μL.[4] Nevertheless, a consensus might be reached on what the lowest qPCR results are that corresponded to the IF-positive samples.[65–67] Therefore, when such a threshold is validated, it will be reasonable to propose only qPCR for PCP diagnosis. At the other end of the spectrum, there is little doubt about the interpretation of qPCR-negative results and a consensus has been reached about the negative predictive value of PCR assays. Thus, a negative qPCR can rule out the diagnosis of PCP in BAL (**A-II**), assuming technical sources of false negatives have been eliminated. Nevertheless, a negative qPCR result cannot exclude the diagnosis in IS, sputa or URS (**A-II**) since the fungal load is lower in these specimens compared with BAL (see above).[3,60,61]

Discrepancies appear with respect to IF-negative qPCR-positive results corresponding either to active infection or carriage. Carriage (or colonization) refers to the presence of the fungus or its DNA in the absence of clinical pneumonia. Some authors have proposed that qPCR quantification could be used to discriminate active pneumonia from carriage based on two thresholds (one to retain and one to exclude PCP) and a grey zone in between without definitive diagnosis.[3,35,36,60] Different thresholds have been proposed based on studies including various populations of patients: HIV-positive[68] or -negative[60] or both.[3,68] However, some authors were not able to discriminate PCP from carriage based on qPCR results.[69] This could reflect a more continuous progression from carriage to active infection in terms of fungal load in respiratory specimens.

Since most of the BAL procedures are performed for investigating pneumonia as determined by signs and symptoms, the detection of *P. jirovecii* DNA could not be regarded as simple carriage even if simultaneous infections (bacteria, virus, fungi, parasites) are documented, which is common in immunocompromised patients. Whether or not a patient with an IF-negative, qPCR-positive result warrants specific therapy when immunosuppression is ongoing is not easy to decide.[66] These matters cannot be solved without appropriate clinical prospective studies. One may nevertheless notice that prophylaxis with trimethoprim/sulfamethoxazole (co-trimoxazole) has resulted in negative PCR results,[4,66,67,70] underlining its effectiveness in preventing PCP disease.

There is little doubt that qPCR has the potential to replace microscopy and IF in the future because of reliability of the results, quantification and low workload when automation becomes possible (nucleic acid extraction and amplification). However, at present, either for technical or economic reasons, numerous laboratories rely only on microscopy. Moreover, as long as thresholds for distinguishing between colonization and infection in non-HIV patients, which can be achieved only using qPCR, are not defined, we cannot propose the definitive replacement of microscopy with qPCR. Thus, no recommendation about the preferential use of qPCR over IF is proposed.

Serum (1–3)-β-D-glucan

β-D-Glucan is a major cell wall polysaccharide of *P. jirovecii* and many other fungi of medical importance, including the most

frequent causes of fungal disease, namely *Aspergillus* spp. and *Candida* spp.; it is consequently considered to be a 'pan-fungal' marker. Different commercial β-D-glucan detection assays have been developed, including the Fungitec-G-test MK assay and the G-test assay (both from Seikagaku Corp., Tokyo, Japan), the β-glucan test Wako (Wako Pure Chemical Industries Ltd, Osaka, Japan) and the Fungitell (Associates of Cape Cod Inc., East Falmouth, MA, USA). Only the last of these assays has a CE marking and US FDA approval. The test performance of serum β-D-glucan detection for the diagnosis of PCP has been evaluated in several studies. Some investigators evaluated this marker in patients with HIV infection, others included mixed populations comprising patients with HIV infection and patients with other types of immunosuppression, and some included only patients without HIV infection.[71,72]

Recently, two meta-analyses were conducted to evaluate the accuracy of serum β-D-glucan detection for diagnosing PCP.[71,72] The meta-analysis of Karageorgopoulos *et al.*[71] included 14 studies, of which two were prospective studies. Here, the diagnosis of PCP was based on the presence of relevant clinical manifestations (not detailed in the article) together with the detection of the pathogen in sputum or BAL fluid, either by microscopy or by PCR. The pooled sensitivity and specificity of β-D-glucan were 94.8% (90.8%–97.1%) and 86.3% (81.7%–89.9%), respectively, and the negative likelihood ratio was 0.06 (0.03–0.11). The area under the summary receiver operating characteristic (AUC–SROC) curve was 0.96 (0.94–0.98). The systematic review and meta-analysis of Onishi *et al.*[72] (12 studies included with two prospective studies) yielded similar results. In this meta-analysis, microscopic visualization of *Pneumocystis* cysts or trophic forms was required to fulfil the case definition. The pooled sensitivity and specificity of β-D-glucan were 96% (92%–98%) and 84% (83%–86%), respectively, and the AUC–SROC was 0.96 (0.94–0.99). The majority of cases had HIV infection but the diagnostic accuracy for these patients and those without HIV infection was not significantly different. Consequently, we recommend the use of β-D-glucan in serum as a contributive laboratory diagnostic tool for the diagnosis of PCP (**A-II**).

Given the high sensitivity of β-D-glucan detection in serum, a negative serum β-D-glucan result can exclude PCP in a patient at risk of the disease (**A-II**). Based on the performance characteristics from the two meta-analyses,[71,72] simulated increasing prevalence rates give different negative predictive values. The negative predictive value varies between 98.5% and 98.9% for a PCP prevalence of 20%. Even at a prevalence rate as high as 50%, the negative predictive value remains very high ($\geq$94%). This makes serum β-D-glucan testing to exclude PCP particularly attractive in patients in whom a BAL test is not feasible. However, its pan-fungal nature and the many other factors that could account for a false-positive β-D-glucan result mean a positive β-D-glucan test cannot be used to confirm the diagnosis of PCP. Instead, a positive β-D-glucan test should trigger an additional diagnostic work-up.

Few studies focused on the follow-up of β-D-glucan kinetics for the assessment of treatment response.[4,66,67,73,74] β-D-Glucan declines slowly in patients with PCP and can persist above the threshold for positivity long after clinical resolution of the original infection.[74] Also, a marked increase in β-D-glucan levels despite clinical improvement was observed in several patients.[73,75–78] The prognostic value of β-D-glucan kinetics within a clinically meaningful time frame remains unclear. Hence, we do not recommend the use of β-D-glucan in serum for the follow-up of PCP treatment (**A-II**).

No recommendation for β-D-glucan detection in BAL fluid was made due to the absence of supporting data.

Genotyping and epidemic investigation

Based on serological survey or DNA detection in healthy infants,[79] almost all infants have encountered *P. jirovecii* before reaching 2 years of age. Normal, healthy individuals are the reservoir of the disease, as extrapolated from transmission experiments from healthy to immunocompromised mice.[80] Airborne transmission is also possible in humans since fungal DNA can be detected in the surrounding air of patients with PCP, with an inverse relationship between fungal burden and the distance of sampling.[81] This has been confirmed by the repeated observation of clusters of infection in renal transplant recipients.[82] The ascus (cyst) could be the infecting agent, as demonstrated in mice.[83] The transmission of *Pneumocystis* to naive immunocompromised hosts results in a progressive increase in the fungal burden in the lungs.[84]

Genotyping based on a multilocus approach was initially developed that utilized single-strand conformation polymorphism (SSCP).[85] Recently, an MLST scheme analysing three markers (cytochrome b, mitochondrial 26S rRNA gene, superoxide dismutase) provided an index of discrimination power (the average probability that two unrelated specimens randomly chosen will be assigned to different types by the method) of 0.987,[86] which is above the 0.95 threshold recommended.[85] This MLST scheme consists of single-round PCRs that are sensitive enough even for samples with low fungal loads.[86] An epidemic can be excluded when different genotypes are observed. Two loci with a 0.957 index of discrimination power (cytochrome b and mitochondrial 26S rRNA gene) can be sufficient to accelerate epidemic investigations (**A-II**).[86] The results of the most discriminating current methods [i.e. internal transcribed spacer (ITS) sequencing with subcloning of the PCR products[87] and multitarget SSCP[88]] suggest that epidemic cases are mostly infected by a single genotype.

The number of *P. jirovecii* MLST genotypes is unknown but is thought to range from 43 using the SSCP method[85] to at least 60 using ITS sequencing methods.[89] Co-infection of a single patient with two or more genotypes has been reported using all typing methods,[90–96] ranging from a few percent using direct DNA sequencing[86,97] to around 70% using SSCP[98] or short tandem repeat typing.[99,100] Geographical variation and other parameters probably account for part of these variations. However, direct sequencing of PCR products is not able to detect low numbers of co-infecting types. Moreover, PCR amplification creates an artificial diversity of genotypes when applied to mixed templates.[101] As a consequence, it is still not clear whether the infection in immunocompromised adults results from recently acquired microorganisms or from reactivation of a hitherto latent infection. A common nomenclature of the various alleles of the markers used for *P. jirovecii* genotyping is needed. To help in attaining this goal a web site has been created that allows identification of alleles (http://mlst.mycologylab.org). However, molecular methods for *P. jirovecii* genotyping are still evolving with implementation of microsatellite-based genotyping methods,[99,100] and new-generation sequencing has been reported that is able

to detect many more co-infecting genotypes than previously reported.[102] Given the present uncertainty about which is the best genotyping marker, we recommend seeking expert laboratory assistance for investigating epidemics.

Drug resistance detection

Trimethoprim/sulfamethoxazole remains the standard drug for prophylaxis and first-line therapy for patients with HIV infection and other immunocompromised patients.[2,40] Whereas trimethoprim seems to inhibit dihydrofolate reductase (DHFR),[103] sulfamethoxazole, like other sulfa and sulfone agents (dapsone and sulfadoxine), inhibits dihydropteroate synthase (DHPS), a key enzyme in *de novo* folic acid synthesis.[104] DHFR synonymous and non-synonymous polymorphisms have been identified,[105,106] but their association with co-trimoxazole exposure is still unclear.[106] DHPS synonymous and non-synonymous polymorphisms have also been identified, and an association between some DHPS non-synonymous polymorphisms and prior sulfa prophylaxis has been detected.[104,107] The studies performed to define the relationship between the use of sulfa and sulfone agents and mutations in their therapeutic targets suggest that there is less selective pressure on DHFR than on DHPS. Non-synonymous SNPs are the most frequent sequence variations in the DHPS locus and they result in amino acid substitutions at positions 55 and 57 (single mutations), or both (combined double mutation). These non-synonymous SNPs are located at the active site of the enzyme and are similar to mutations leading to sulfa drug resistance in other microorganisms,[108,109] suggesting that they do confer some level of resistance.

DHPS mutations at codons 55 and 57 are associated with failure of co-trimoxazole prophylaxis in patients with PCP and AIDS, suggesting that these mutations are selected by drug pressure.[109,110] However, mutations have also been detected in PCP patients and in those carrying *P. jirovecii* who were not receiving sulfa or sulfone agents at the time. Therefore, rather than selection pressure by sulfa prophylaxis, the presence of DHPS mutations could be explained by incidental person-to-person transmission and may serve as an epidemiological marker.[111] Nonetheless, sulfa agents given at therapeutic doses do result in successful treatment in most cases of PCP harbouring these DHPS mutations, casting doubt on the clinical significance of this finding.[112] Consequently, we do not recommend the routine detection of DHPS mutations in case of treatment failure (**B-II**).

Conclusions

Microscopy after conventional or IF staining presents a sensitivity too low to be reliably used to diagnose PCP in patients with haematological malignancies as the disease is associated with much lower fungal loads than are found among patients with PCP and HIV infection. The evidence-based review of currently available PCR assays strongly supports their use for the diagnosis of PCP (**A-II**). To avoid false-positive results due to contamination of the environment with PCR products, qPCR without opening the reaction tubes is strongly recommended (**A-II**). *P. jirovecii* qPCR assays should be harmonized as proposed for *Aspergillus* qPCR (European *Aspergillus* PCR Initiative, www.eapcri.eu). BAL is the preferred specimen (**A-II**) for PCP diagnosis and for the diagnosis of other respiratory pathogens in immunocompromised patients. However, there is presently no clinical cut-off either for distinguishing carriage from infection in haematology patients or for defining a strict concordance between IF staining and quantitative PCR results. These thresholds could be refined in additional studies focusing on populations that are as homogeneous as possible. Currently, a negative PCR result in BAL is strongly associated with the absence of PCP (**A-II**), whereas this is not the case for other respiratory specimens. β-D-Glucan detection in serum represents a promising test since a negative result is strongly associated with the absence of PCP (**A-II**). However, the optimal cut-offs for positivity need to be better defined and clinically validated. A simultaneous search for β-D-glucan and DNA in serum could help to improve the specificity of β-D-glucan assays.[113] A specialized centre should be contacted to assist with investigations for epidemiology and drug resistance. The current recommendations apply to adults only since additional studies are needed for paediatric populations.

Acknowledgements

We thank Patricia Munoz (Madrid) and J. Peter Donnelly (Nijmegen) for their helpful support of the plenary debate of this guideline at ECIL-5. We also thank Jean-Michel Gosset and the staff of KOBE, group GL-Events, Lyon, France, for the organization of the meeting.

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Funding

The ECIL-5 meeting was supported by unrestricted educational grants from Astellas Pharma, Gilead Sciences, Merck, and Pfizer.

Transparency declarations

S. B. received a consultant honorarium from Myconostica. The remaining authors have none to declare.

Author contributions

The content of the manuscript was developed by all authors. A. A., P. H., K. L., W. M., J. H. L., O. M. and S. B. drafted the manuscript and all authors approved the final version.

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